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Purification of Glucose-6-Phosphate Dehydrogenase from Baker's Yeast in Aqueous Two-Phase Systems with Free Triazine Dyes as Affinity Ligands

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Abstract

To improve the selectivity of glucose-6-phosphate dehydrogenase (G6PDH) extraction by an aqueous two-phase system, a simple and inexpensive affinity aqueous two-phase system using unbound reactive triazine dyes as ligands was introduced. In a polyethylene glycol (PEG)/hydroxypropyl starch (PES) system, the unbound free triazine dyes, Cibacron Blue F3GA and Procion Red HE3B, partitioned unevenly in the top PEG-rich phase and thus showed an affinity effect on G6PDH, but no influence on hexokinase. The various parameters investigated were pH of the system, buffers, molecular weight of PEG, and ligand type and concentration. A two-step affinity extraction process was established for the purification of G6PDH from baker's yeast. The total yield of G6PDH was 66.9% and purification factor was 2.35.

Index Entries : Aqueous two-phase systems; affinity partitioning; triazine dyes; glucose-6-phosphate dehydrogenase; baker's yeast.

Introduction

Several procedures for the rapid purification of enzymes from yeast and other microorganisms have been developed, mainly to avoid undesirable proteolytic degradation of the target enzymes in the course of their

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isolation. In particular, the introduction of preparative affinity chromatography (using natural ligands or artificial dye ligands) has led to significant improvement in the efficiency and economy of time for these procedures *(1)*. However, when large-scale preparations are required, various microbiological and technological problems arise and often limit the use of water-insoluble hydrophilic or hydrophobic resins *(2)*. Aqueous two-phase systems are finding increasing application in the recovery of proteins *(3,4)*. Moreover, the development of affinity partitioning for the purification of proteins combined the advantages of biospecific adsorption of selective compounds and the ability to work in a homogeneous phase *(5,6)*.

Affinity partitioning as currently practiced requires the ligand to be covalently attached to one of the phase-forming polymer components, thereby causing the ligand/polymer to partition predominantly into one of the phases. Although triazine dyes covalently coupled to polyethylene glycol (PEG) can be produced on a large scale, the process is complicated and requires a chromatographic step and several organic solvent extractions *(7)*. To simplify the affinity partitioning, Giuliano *(8)* used the free dyes, uncoupled to the phase-forming polymers, as affinity ligands for the partitioning of lysozyme in a polyvinylpyrrolidone/maltodextrin aqueous two-phase system. In the PEG/phosphate system, it was reported that free triazine dyes, partitioned predominantly into polymer phase, showed the affinity effect on some dehydrogenases and kinases *(9,10)*. Lin et al. *(11,12)* also used free triazine dyes in affinity extraction of lactate dehydrogenase in a PEG/hydroxypropyl starch (PES) aqueous two-phase system.

Glucose-6-phosphate dehydrogenase (G6PDH), the first enzyme in the pentose phosphate pathway, is widely distributed in nature. It is a part of the antioxidant enzymatic system that has an important role in tissue protection against the destructive action of oxygen-free radicals *(13)*. With hexokinase, G6PDH presents great interest as analytical reagents for the measurement of creatin-kinase activity, adenosine triphosphatase (ATP), and hexose concentrations *(14)*.

In this study, we investigated the partition of G6PDH with the free Procion Red HE3B and Cibacron Blue F3GA, unbound to phase-forming polymer, as affinity ligands in PEG/PES and PEG/phosphate aqueous two-phase systems. The effects of various parameters, such as the pH of the system, molecular weight of PEG, buffer solution, and concentration of ligands, on partition coefficients of the enzymes were systematically studied. A two-step method for the purification of G6PDH from baker´s yeast was proposed for the first time.

Materials and Methods

Chemicals

PEG3000, PEG4000, and PEG6000 were purchased from LABSYNTH Produtos para Laboratorios Ltda. Hydroxypropyl starch (Reppal PES100) was a kind gift of Professor J. A. Teixeira (Departmento de Engenharia

Biologica, Universidade do Minho, Portugal). Cibacron Blue F3GA and Procion Red HE3B were purchased from Sigma. G6PDH, hexokinase, β-nicotinamide adenine dinucleotide phosphate (NADP), ATP, D-glucose-6-phosphate, and D-glucose were also from Sigma. All other reagents were of analytical grade.

Commercial pressed baker´s yeast (*Saccharomyces cerevisiae*) was used. The cells were suspended in 50 m*M* Tris-HCl buffer (pH 7.5) containing 5 m*M* $MgCl_2$, 10 m*M* β-mercaptoethanol, and 2 m*M* phenylmethylsulfonyl fluoride.

Aqueous Two-Phase Systems

The systems were prepared from stock solutions: 50% (w/w) PEG, 30% (w/w) PES, 40% (w/w) ammonium sulfate, and 40% (w/w) phosphate (mixture of NaH_2PO_4 and K_2HPO_4). The total weight was 3.0 g for the polymer/polymer (PEG/PES) system, and 4.0 g for the polymer/salt system. All concentrations were given in weight percentage except as indicated otherwise.

Cell Disruption

A volume of 100 mL of glass beads (0.5 mm diameter) and 100 mL of Tris-HCl cell suspension were introduced into the 250 mL chamber of a vertical cell disrupter *(15)*. Cell disintegration was carried out at 5°C under agitation of 2300 rpm, and then the disrupted cell suspension was centrifuged at 18,000*g* for 30 min at 5°C. The supernatant was used in further experiments.

Partitioning Experiments

The enzymes or the supernatant was added to the systems. After 15 min of inversion mixing to ensure partition equilibrium, phase separation was accomplished by centrifuging at 2880*g* for 5 min, and samples were withdrawn for analysis. The experiments were conducted at room temperature for pure enzymes and at 4°C for disrupted cell supernatant.

Preparation of Dye Derivatives

According to Giuliano *(8)*, the amino derivatives of chlorotriazine dyes (Cibacron Blue F3GA and Procion Red HE3B) were prepared as follows: Four grams of the dye was first dissolved in 50 mL of methanol followed by the addition of 20 mL of concentrated ammonium hydroxide. The solution was refluxed for 40 min. Solvents were removed via rotary evaporation at reduced pressure (vacuum of 450 mmHg). The remaining solid was washed with 300 mL of acetone and dried for use. Acetone was recovered by rotary evaporation.

Analyses

G6PDH and hexokinase activity was measured spectrophotometrically at 30°C by following the rate of $NADP^+$ reduction at 340 nm in a

coupled enzyme assay system *(16)*. For both enzymes, one unit of enzyme activity was defined as the amount of enzyme required to form 1.0 μmol of NADPH/min with the substrate in excess. The partition coefficient of the enzymes (K_e) was defined as the ratio of the enzyme activities in the top and bottom phases.

The concentration of protein was determined by Bradford´s *(17)* method using bovine serum albumin as standard. The concentration of the dye and the derivatives was determined photometrically at 615 nm for Cibacron Blue F3GA and its derivative, and 515 nm for Procion Red HE3B and its derivative. The partition coefficient of the dyes (K_L) was defined as the ratio of the concentrations in the top and bottom phases.

The binding of a ligand to an enzyme was evaluated by inhibition constant, K_i, which is the dissociation constant of the enzyme-ligand complex. This value was determined by Lineweaver-Burk plot. To calculate K_i, it was assumed that the inhibition of the dyes to G6PDH and hexokinase is competitive; thus, we obtained the linear relationship of $1/A \sim [I]$, in which A is the activity of enzymes and $[I]$ is the concentration of dyes. With the intercept and slope of $1/A \sim [I]$, the K_i was calculated.

Enzymatic assay was carried out in the corresponding equilibrated top and bottom phases.

Results and Discussions

Partitioning of Dyes in Aqueous Two-Phase System

Usually, the ligand was covalently coupled to the target phase polymer in the affinity aqueous two-phase system, so as to confine the ligand in one phase. To use uncoupled free dye molecules as ligand, their partition behaviors in the system need to be estimated first.

Partitioning in PEG/Salt Systems

In PEG/salt aqueous two-phase systems, the dyes partitioned predominantly in the top PEG phase. The effect of pH on the partitioning of Cibacron Blue F3GA and Procion Red HE3B in the PEG/phosphate system is presented in Table 1. With the increase in pH, the partition coefficient of both dyes increased. This may be caused by the repulsion of negatively charged phosphate, because of more negative charges on the dye molecules in the higher pH range. The effect of molecular weight of PEG on the partition coefficient of Cibacron Blue F3GA and Procion Red HE3B in the PEG/ammonium sulfate system is presented in Table 2. With the increase in molecular weight of PEG, the partition coefficient of both dyes increased, which is different from the behavior of biopolymers *(3)*.

Partitioning in PEG/PES System

In the PEG/PES system, although the partition coefficients are smaller than in the PEG/salt system, most of the dyes still partitioned in the top PEG phase, i.e., the base for the affinity partitioning of the enzymes. For affinity partitioning, the affinity ligands should have uneven distribution

Table 1
Effect of pH on Partitioning of Dyes
in PEG3000 (12.5% [w/w])/Phosphate (10% [w/w]) System

	K_L	
pH	Cibacron Blue F3GA	Procion Red HE3B
3.45	7.3	6.3
5.97	319	310
7.48	707	>1000
9.70	>1000	>1000

Table 2
Effect of Molecular Weight of PEG
on Partitioning of Dyes in PEG3000 (12.5% [w/w])/
Ammonium Sulfate (10% [w/w]) Systems

Molecular	K_L	
wt (Daltons)	Cibacron Blue F3GA	Procion Red HE3B
3000	16.5	37.8
4000	27.6	133
6000	227	741

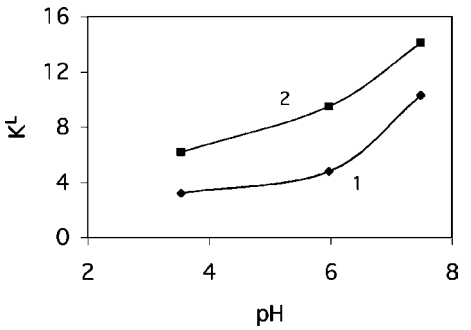

Fig. 1. Effect of pH on partitioning of Cibacron Blue F3GA (1) and Procion Red HE3B (2) in PEG3000 (8.3% [w/w])/PES100 (15% [w/w]) system: phosphate buffer (2.67% [w/w]).

in the aqueous two-phase system. If the affinity ligands partition evenly in the two phase, no affinity effect will present.

Figure 1 shows the effect of pH on the partitioning of Cibacron Blue F3GA and Procion Red HE3B in the PEG/PES system. With the increase in pH, the partition coefficients of both dyes increased, which may also be attributed to the more negative charges on dye molecules in higher pH condition. Most of the buffer phosphate partitioned in the bottom PES phase.

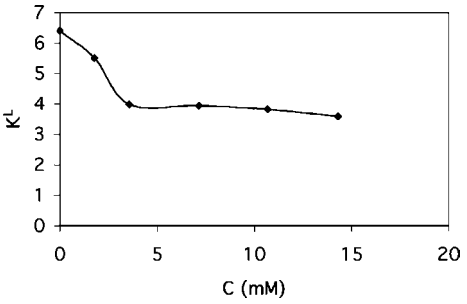

Fig. 2. Effect of Tris-HCl buffer on partitioning of blue dye in PEG6000 (8.3% [w/w])/PES100 (15% [w/w]) system: pH 7.5.

Although the higher partition coefficient was needed for affinity partitioning, the pH should not exceed 7.5 because of the instability of the enzymes. The influence of Tris-HCl buffer (pH 7.5) on the partitioning of Cibacron Blue F3GA in the PEG/PES system was investigated (Fig. 2). At the beginning, the increase in Tris-HCl concentration decreased the partition coefficient of the blue dye. A further increase had almost no effect on the blue dye partitioning.

Effect of Molecular Weight of PEG on Partitioning of Enzymes

The effect of molecular weight of PEG on the partition coefficient of G6PDH and hexokinase activity is shown in Fig. 3. An increase in molecular weight of PEG brought about a reduction in partition coefficient of G6PDH and hexokinase. This may be caused by the excluded volume effect of PEG *(3,9)*, and the different tie-line length of the systems.

Table 3 shows the effect of molecular weight of PEG on the partition coefficient of G6PDH in the PEG/PES100 system with and without blue dye ligand. In the PEG6000 system, the partition coefficient was enhanced from <1 without ligand to >1 with the blue dye ligand.

Effect of Buffer Solution on Partitioning of Enzyme

The effect of Tris-HCl concentration on the partitioning of G6PDH in the PEG6000/PES100 system with and without blue dye ligand was estimated (Fig. 4). Generally, most negatively charged proteins partitioned to the top PEG phase in the PEG/PES aqueous two-phase system. In the presence of Tris-HCl buffer, the proteins would be transferred to the bottom PES phase. With proper ligand, the G6PDH could be transferred to the top phase again by the selective affinity effect, and the other proteins might stay in the bottom phase. As can be seen, both partition coefficients, with and without ligand, decreased with the increase in Tris-HCl concentration. However, at a concentration of about 9 m*M*, the G6PDH was transferred from the bottom phase ($K_e < 1$) to the top phase ($K_e > 1$) by the blue dye ligand.

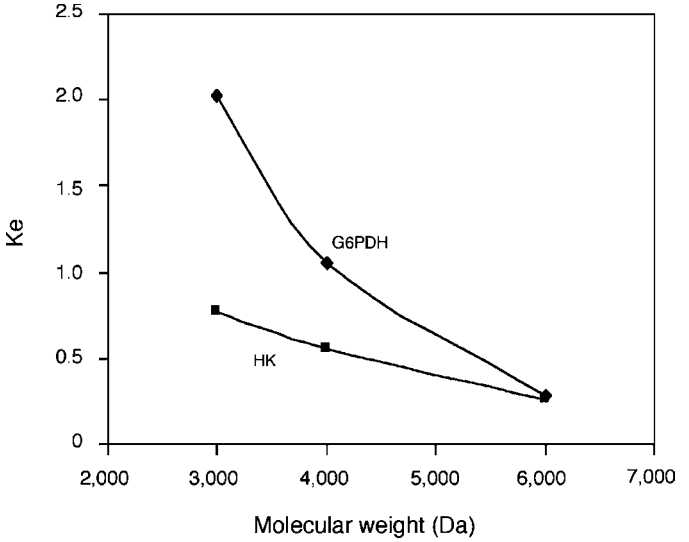

Fig. 3. Effect of molecular weight of PEG on partition coefficient of enzymes in PEG (8.3% [w/w])/PES100 (15% [w/w]) system: phosphate (2.67% [w/w]), pH 7.3. HK, hexokinase.

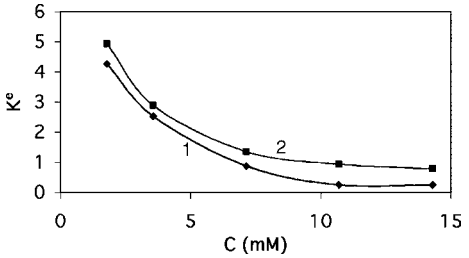

Fig. 4. Effect of Tris-HCl buffer on the partition coefficient of G6PDH in PEG6000 (8.3% [w/w])/PES100 (15% [w/w]) system: pH 7.5, without (1) and with (2) 0.067% blue dye.

Table 3
Effect of Molecular Weight of PEG on Partition Coefficient of G6PDH in PEG/PES100 System[a]

Molecular wt (Daltons)	K_e		ϕ^b
	No dye	Cibacron Blue F3GA	
3000	4.46	4.96	1.11
6000	0.73	1.59	2.18
10,000	0.2	0.34	1.7

[a]PEG (8.3% [w/w])/PES100 (15.0% [w/w]) system; 1.33% phosphate (buffer), 0.033% blue dye, pH 7.3.

[b]Ratio of partition coefficient in the presence to that in the absence of dye.

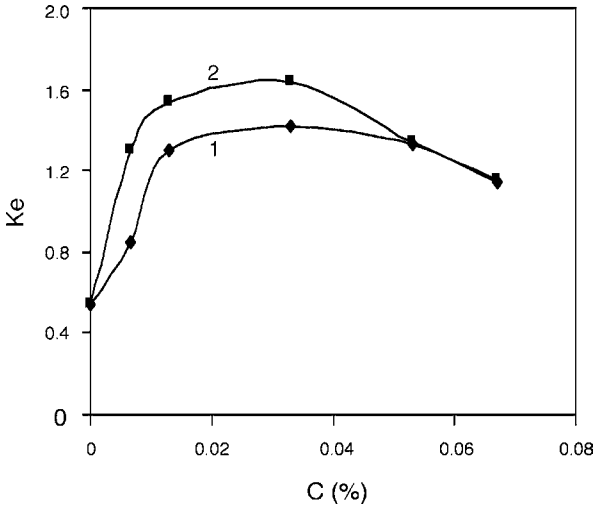

Fig. 5. Effect of dye concentration on the partitioning of G6PDH in PEG6000 (8.3% [w/w])/PES100 (15% [w/w]) system: 9 m*M* Tris-HCl, pH 7.5; (1) Cibacron Blue F3GA; (2) Procion Red HE3B.

Effect of Ligand Type and Concentration on the Partitioning of Enzyme

Figure 5 shows the effect of Procion Red HE3B and Cibacron Blue F3GA concentration on the partitioning of G6PDH in the PEG6000/PES100 system. The red dye had a stronger affinity effect on the partitioning of G6PDH than the blue one. Initially, when the ligand concentration was low, increasing the dye concentration made more sites available for enzyme binding and the partition coefficients increased rapidly. However, when the dye concentration reached a certain quantity for a given enzyme concentration, a further increase in dye concentration had no effect on the partition coefficients, which is similar to what has been observed when dye-PEG *(18)* or dye-dextran *(19)* was used. A further increase in dye concentration resulted in a decrease in G6PDH partition coefficient and may be induced by a static electric repulsive effect between the same negatively charged dye ligands and the enzyme.

The triazine dyes may form covalent linkage with the proteins. To eliminate the possibility of covalent modification of hexokinase or G6PDH, the reactive chlorine atom of the dyes could be removed by hydrolysis *(20,21)*. According to Giuliano *(8)*, Cibacron Blue F3GA and Procion Red HE3B were converted to their amino derivatives to prevent the formation of dye/protein covalent linkage. The affinity effect of triazine dye ligands and their ammonia derivatives on the partitioning of G6PDH in the PEG 6000/PES100 system is summarized in Table 4. The ligands could enhance the partition coefficient of G6PDH, and the effect of red dye was stronger than that of blue dye.

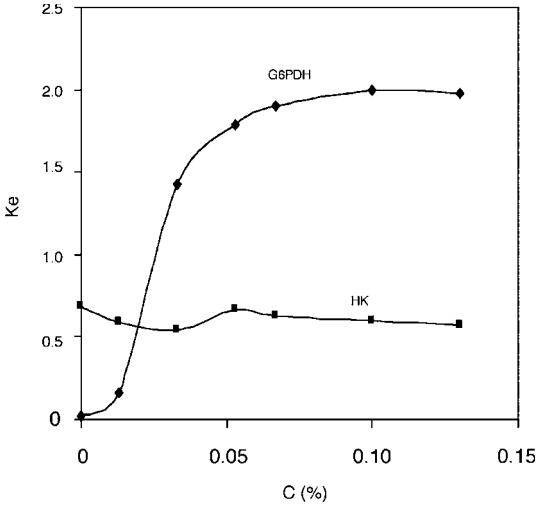

Fig. 6. Effect of Procion Red HE3B concentration on the extraction of G6PDH from baker´s yeast in PEG3000 (8.3% [w/w])/PES100 (15% [w/w]) system: 9 m*M* Tris-HCl, pH 7.5. HK, hexokinase.

Table 4
Effect of Triazine-Reactive Dyes on Partitioning of G6PDH[a]

Dye ligand	K_L	K_e	ϕ^b
No dye		0.54	
Red	8.35	1.64	3.0
Red derivative	8.34	1.60	3.0
Blue	3.90	1.42	2.6
Blue derivative	3.93	1.44	2.7

[a]PEG6000 (8.3% [w/w])/PES100 (15% [w/w]) system; 9 m*M* Tris-HCl, pH 7.5, 0.033% dyes.

[b]Ratio of partition coefficient in the presence of dye to that in the absence of dye.

Purification of G6PDH from Baker´s Yeast

Partitioning of Enzymes from Baker´s Yeast

At 4°C, the PEG6000 system became too viscous to handle so the PEG3000 system was chosen to investigate the partitioning behavior of the enzymes in disrupted cell supernatant. Figure 6 indicates that the effect of Procion Red HE3B concentration on the partitioning of G6PDH and hexokinase in the PEG3000/PES100 system. The red dye ligand enhanced the partition coefficient of G6PDH and showed no affinity effect on hexokinase. The shape of the curve for G6PDH is similar to that of pure enzyme (Fig. 5), but one point should be noted: the concentration of dye ligand is higher for the affinity partitioning of G6PDH. In disrupted cell supernatant, there are many other enzymes that may bind the red dye ligand, so higher ligand concentration is needed for the affinity partitioning of G6PDH.

Table 5
Effect of Triazine-Reactive Dyes on Partitioning of Enzymes[a]

		K_e		ϕ^b	
Dye ligand	K_L	G6PDH	Hexokinase	G6PDH	Hexokinase
No dye		0.017	0.68		
Red	6.95	1.9	0.63	112	0.93
Red derivative	7.02	1.9	0.65	112	0.96
Blue	2.84	1.4	0.97	82	1.40
Blue derivative	2.85	1.5	1.00	88	1.50

[a]PEG3000 (8.3% [w/w])/PES100 (15% [w/w]) system; 9 m*M* Tris-HCl, pH 7.5, 0.10% dyes.
[b]Ratio of partition coefficient in the presence to that in the absence of dye.

The affinity effect of triazine dye ligands and their ammonia derivatives on the partitioning of G6PDH and hexokinase in the PEG3000/PES100 system is summarized in Table 5. The ligands could enhance the partition coefficient of G6PDH greatly. Without the dye ligand, the G6PDH was concentrated in the bottom phase ($K_e = 0.017$). With the red dye ligand, most of the G6PDH partitioned into the top phase ($K_e = 1.9$), but for pure G6PDH, the partition coefficient changed from 0.54 to 1.64 by the red dye ligand (Table 4).

To elucidate the mechanism (different effect of different dye ligands—blue and red) of affinity aqueous two-phase partitioning of proteins (G6PDH and hexokinase), Flanagan and Barondes *(22)* had proposed a thermodynamic model (Eq. 1). This model describes the distribution equilibrium of proteins in the affinity aqueous two-phase systems. It relates the partition coefficient of proteins in the presence of affinity ligands with the partition coefficient in the absence of affinity ligands, and partition coefficient of ligands and the dissociation constants of ligands with protein molecules in the top and bottom phases. The equation was deduced from Gibbs' free energy in equilibrium state:

$$K = K_0 \left(K_L K_{ib} / K_{it}\right)^a \tag{1}$$

in which K and K_0 are the partition coefficients of proteins in the presence and absence of the ligand, respectively; K_L is the partition coefficient of the ligand polymer; K_{it} and K_{ib} are the dissociation constants of the ligand with protein molecule in the top and bottom phases, respectively; and α is the number of ligand polymer molecules bound per protein molecule.

The binding strength of G6PDH to the dye ligands was similar (similar K_i) in the top and bottom phases (Table 6), but most of the dye partitioned in the top PEG phase, so G6PDH could be brought from the bottom to the top phase by the ligand. The K_L of red dye was higher than that of blue dye, and therefore the affinity effect was stronger. The binding strength of hexokinase to blue dye ligand was weaker than that of G6PDH (larger K_i value),

Table 6
Inhibition Constants of G6PDH and Hexokinase in Disrupted Cell Supernatant by Triazine Dyes[a]

	K_i (10^4 m*M*)			
	Cibacron Blue F3GA		Procion Red HE3B	
Enzyme	In presence of top phase	In presence of bottom phase	In presence of top phase	In presence of bottom phase
G6PDH	5.22	4.56	3.99	1.41
Hexakinase	193	236	71.3	3.20

[a]In top or bottom phase of PEG3000 (8.3% [w/w])/ PES100 (15% [w/w]) system; 9 m*M* Tris-HCl, pH 7.5.

Table 7
Partitioning of G6PDH in PEG3000 (12.5% [w/w])/ Phosphate (10% [w/w]) System[a]

Dye	K_e
Cibacron Blue F3GA	0.0043
Procion Red HE3B	0.0040

[a]pH 7.48; 0.05% dyes.

so the affinity effect was weakened. For red dye ligand, the binding strength of hexokinase in the bottom phase was stronger than that in the top phase, and no affinity effect was observed.

Two-Step Method for Purification of G6PDH from Baker´s Yeast

Table 7 lists the partition coefficients of G6PDH in the PEG3000/phosphate system in the presence of the dye ligands. Although the dyes were concentrated in the PEG-rich top phase (Table 1), the enzyme stayed in the salt-rich bottom phase. The dyes and the G6PDH could be separated in the PEG/phosphate system, indicating that no covalent linkage formed in the experimental condition.

To achieve purification of G6PDH from baker´s yeast, a two-step procedure based on the aforementioned results was developed (Fig. 7). The experiment was performed in a separatory funnel and the system was enlarged to 15 g.

The disrupted cell supernatant was added into the PEG3000/PES100 system (first step). G6PDH was extracted into the top PEG phase by Procion Red HE3B ligand. After phase separation, the top PEG phase was combined with phosphate solution, forming a PEG/phosphate two-phase system (second step). The enzyme was recovered in the phosphate-rich bottom phase. After the "two-step" extraction, the total recovery of G6PDH was 66.9% with a purification factor of 2.35.

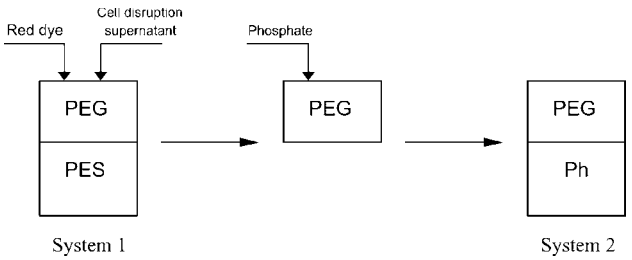

Fig. 7. Diagram of two-step method for purification of G6PDH from baker´s yeast: System 1: PEG3000 (8.3% [w/w])/PES100 (15% [w/w]), 9 m*M* Tris-HCl, pH 7.5; system 2: PEG3000 (11% [w/w])/phosphate (18% [w/w]), pH 7.3.

Conclusions

The partitioning of G6PDH and hexokinase in PEG/PES and PEG/phosphate aqueous two-phase systems was investigated with free triazine dyes, Cibacron Blue F3GA and Procion Red HE3B, as their affinity ligands.

The dyes, not bound to phase-forming polymers, were concentrated in top PEG phase in PEG/phosphate and PEG/ammonium sulfate systems. In the PEG/PES system, although the partition coefficients were smaller than in the PEG/salt system, most of the dyes still partitioned in the top PEG phase, which is the fundamental requirement for the affinity partitioning of the enzymes.

In the PEG/phosphate system, the dyes and enzyme were concentrated in the top PEG and bottom PES phase, respectively. In the PEG/PES system, Procion Red HE3B changed the partition coefficient of pure G6PDH from 0.54 to 1.64 with Tris-HCl buffer. For G6PDH in disrupted cell supernatant, the partition coefficient increased from 0.017 to 1.9 with the red ligand. The enzyme was transferred from the PES-rich bottom phase to the PEG-rich top phase. To purify G6PDH from baker´s yeast, a two-step procedure was proposed. The total yield was 66.9% with a purification factor of 2.35. Thus, a simple, effective affinity partitioning method, with the free dye as ligand for the purification of G6PDH from baker´s yeast was developed.

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